USE OF METALLIC CATIONS TO IMPROVE FUNCTIONAL ACTIVITY OF ANTIBODIES

The present invention pertains to the use of metallic cations, especially divalent or trivalent cations, of Zinc, Copper, Cadmium or Iron in particular, to improve the functional activity of antibodies. More particularly, the subject-matter of the invention is pharmaceutical antibody compositions comprising divalent or trivalent metallic cations.

INTRODUCTION AND PRIOR ART

Passive immunotherapy, much used, is based on administering antibodies, for example monoclonal antibodies, directed against a cell or given substance. Passive immunotherapy using monoclonal antibodies has given encouraging results. However, while the use of monoclonal antibodies offers several advantages, such as assured product safety for example regarding the absence of infectious contamination, it may on the other hand prove to be difficult to obtain an efficient monoclonal antibody.

The risk exists of developing a monoclonal antibody which proves to be little effective and for which side effects are found incompatible with its use in clinical therapy. These two aspects are closely related bearing in mind that low active antibodies are administered in high doses to offset their low active and to achieve therapeutic response. The administering of high doses not only induces side effects, but economically it is also scarcely profitable.

These are major problems for the industrial development of chimeric, humanised or human monoclonal antibodies. By way of example, Protein Designs Labs has suspended phase I/II clinical trials of Remitogen® which is an anti-HLA-DR antibody finding possible use in the treatment of positive, class II MHC cell cancers, in particular B and T lymphocyte leukaemias.

Therefore, one of the objects of the invention is to provide new products or methods with which to overcome the

drawbacks encountered during the industrial development of antibodies, namely their low efficacy and high cost.

Current research focuses on the Fc region of immunoglobulin (Ig) to improve the functional properties of antibodies. Over the longer term, this should allow antibodies to be obtained which bind with and activate the receptors of effector cells (monocytes/macrophages, B lymphocytes, NK and dendritic cells), in more efficient manner. The Fc region of IgGs consists of 2 globular domains called CH2 and CH3. The 2 heavy chains interact closely at the CH3 domains, whilst at the CH2 domains the presence on each of the 2 chains of an oligosaccharide bound to Asn 297 (Kabat numbering) contributes towards the distancing of the 2 CH2 domains. Also, the CH2 and CH3 domains of one same chain are separated by a flexible region defining an interface between the 2 domains.

The interface between the CH2-CH3 domains has been described as being a common fixing site for numerous (glyco)proteins such as FcRn, rheumatoid factors (Corper *et al*, 1997) and some bacterial and viral proteins such as protein A of *Staphylococcus aureus* (Deisenhofer, 1981), protein G of group G *Streptococcus* (Sauer-Eriksson *et al*, 1995), the gE-gI complex of Herpes simplex 1 virus (Chapman *et al*, 1999) and the "core" protein of hepatitis C virus (Maillard *et al*, 2004).

Also, it has been shown that some residues of amino acids, located at this interface in the CH2 domain or CH3 domain, are involved in the fixing to FcRn, to protein A etc. Therefore, human IgGIs mutated at the histidine 435 residue(His 435, Kabat numbering) lose their capability of fixing FcRn and protein A, but maintain their ability to fix FcγRIII (Firan *et al*, 2001; Shields *et al*, 2001).

On the other hand, none of the studies notifying changes of the CH2-CH3 interface of human or murine IgG has shown any relationship between the structure of this part of the IgG molecule and the effector capacities of these IgGs via the FcγR receptors (FcγRI, FcγRII, FcγRIII).

Under the invention, we have determined the three-dimensional structure of the Fc regions of different monoclonal antibodies having different functional activities, ADCC activity in particular and the induced production of cytokins. We have discovered the presence of a zinc ion located between the CH2 and CH3 domains, more precisely bound to residues located at the interface of the CH2-CH3 domains involved in recognition of the Fc region of the antibody by the FcRn receptor, and in the fixing of protein A derived from the bacterial wall of *Staphylococcus aureus*.

Through its location at the interface of the CH2 and CH3 domains, this zinc atom plays an important role in the general conformation of Fc and thereby allows an improvement in Fc binding with its FcγR receptors.

Our research on the three-dimensional structure of the Fc region of these antibodies has revealed that the presence of metallic cations such as Zinc, Copper, Cadmium or Iron, in the crystallisation solution is always associated with the so-called open conformation of the Fc region of IgGs (Radaev *et al.*, 2001). This open conformation promotes the fixing to FcγR receptors, to FcγRIII in particular. Therefore, it is henceforth possible to potentialise the functional activity of monoclonal or polyclonal antibodies through the use of metallic cations. On the contrary, if the fixing of metallic cations such as zinc, copper, cadmium or iron to the antibodies is abolished, especially at the CH2-CH3 interface, through the use of mutants of the peptide sequence or suitable chemical substances, this leads to obtaining antibodies whose functional properties are cancelled or strongly reduced.

Therefore the invention provides a universal, economic solution to solve the problems related to the low efficacy of monoclonal antibodies currently available or being developed, through the use of metallic cations which improve the functional activity of antibodies. For this purpose, we propose a method for potentialising the functional activity of antibodies by means of metallic cations. Finally, through the modification of the binding site to the metallic cations, the

invention also provides class IgG1 antibodies having a reduced capacity to activate the FcγRIII receptor, and class IgG3 antibodies artificially possessing a fixation site for a metallic cation.

DESCRIPTION

Therefore, a first subject of the invention is the use of divalent or trivalent metallic cations to improve the functional activity of antibodies.

Our three-dimensional studies of the Fc region of antibodies have revealed that the presence of metallic cations such as Zinc, Iron, Copper or Cadmium is always associated with the so-called open conformation of the Fc region of IgGs. This open conformation promotes the fixing of antibodies to the FcγR receptors, in particular to FcγRIII. It is therefore henceforth possible to potentialise the functional activity of monoclonal or polyclonal antibodies by means of said metallic cations.

Preferably, the metallic cation used is zinc. Our analyses have enabled us to evidence the presence of a zinc ion bound to the histidine 310 and histidine 435 residues (in the present application, the numbering used is Kabat numbering, Kabat database http://immuno.bme.nwu.edu).

Through its location on the Fc region, this zinc atom plays a major role in the general conformation of the Fc region, thereby enabling improved binding of the Fc region to its receptors.

Advantageously, therefore, these cations are used to interact with the Fc region of the antibodies so as to take part in stabilising this region.

More particularly, they are used to participate in controlling the opening of the Fc region of antibodies and thereby to promote maintaining of the so-called "open" conformation of the antibodies i.e. the maintaining of a certain distance between the CH2 domains to promote fixing of the Fc region to its receptors. The presence of the metallic

cations can induce opening of the Fc region even if the metallic cation is not maintained in its site.

Therefore, advantageously, these metal ions promote the fixing of antibodies to the FcγR receptors, in particular to the FcγRIII receptor.

In addition, the metallic cation, according to another characteristic of the invention, can promote the drawing together of several antibody Fc regions via a second fixing site involving the histidine 268 and 285 residues (Kabat numbering) facilitating the activation of FcγRs, more particularly FcγRIIIs and transduction as soon as signalling is given via these receptors.

By "functional activity" is meant, in non-limitative manner, ADCC activity (Antibody-Dependent Cell-mediated Cytotoxicity), CDC activity (Complement Dependent Cytotoxicity), phagocytosis activity, endocytosis activity or the induction of cytokin secretion. Therefore, the use of metallic cations such as described in the invention makes it possible to improve functional activity by at least 50%, preferably 60%, or 70%, 80%, 100%, preferably by 200% or 300%.

By "receptors" is meant not only the molecules of FcγR such as FcγRIII present on the cells of the immunity system such as the monocytes, macrophages, B and T lymphocytes, NK cells and dendritic cells, but also the FcRn, the complement molecules such as Clq and those of the bacterial walls such as protein A.

By "antibody" is meant any monoclonal or polyclonal antibody. If the antibody is a monoclonal antibody, it may be chimeric, humanised or human. Advantageously, this antibody is an IgG, for example an IgG1 or IgG3, especially human. In addition, the term antibody includes any glycoprotein containing a Fc region, e.g. human, and one more fragments, domains or antibody derivatives. By "antibody domain" is meant any of the domains VL, CL, VH, CH1, CH2, CH3, CH4. By "antibody fragment" is meant any fragment which contains a complete fixing site for an antigen, chosen from among the fragments Fv, scFv, Fab, Fab', F(ab')2, and by "antibody

derivative" is meant any antibody able to comprise one or more mutations, substitutions, deletions and/or additions of one or more residues of amino acids, and multispecific and polyfunctional antibodies.

By "divalent or trivalent metallic cation" or by "metallic cation" is meant any metallic cation of oxidation state +2 or +3 and more particularly zinc, iron, copper, cadmium, cobalt, nickel, manganese, gallium, gadolinium, selenium, gold, platinum, palladium or an analog. Preferably, these metallic cations are zinc, iron, copper or cadmium, and in particularly advantageous manner it is zinc. By "analog" is meant any free or bound ion able to bind at the Fc region of antibodies, and more particularly to residues His 310, His 435, the Asn 434 and His 433 residues (Kabat numbering) possibly also taking part in this fixing.

A second subject-matter of the invention is a method for potentialising the functional activity of antibodies via the Fc region, comprising a step consisting of adding a suitable quantity of at least one metallic cation to the biological system producing the antibodies, or to a solution comprising antibodies before and/or after purification, or to the preserving solution, or to the end formulation in the form of an injectable solution of antibodies.

Advantageously these metallic cations are zinc, iron, copper or cadmium.

By "biological system" is meant cell lines, non-human transgenic plants or animals. Among the cells, the cells chosen may be derived from cell lines transfected with a vector comprising the gene encoding said antibody, for example eukaryote or prokaryote cells, in particular cells of mammals, insects, plants, bacteria or yeasts. More specifically, it is possible to use rat myeloma cells such as YB2/0.

It is also possible to use CHO cells, in particular CHO-K, CHO-Lec10, CHO-Lec1, CHO Pro-5, CHO dhfr- or other cells lines from among Wil-2, Jurkat, Vero, Molt-4, COS-7, 293-HEK, K6H6, NSO, SP2/0-Ag 14 and P3X63Ag8.653, PERC6 or BHK.

Preferably, a molar concentration of zinc is added at least equal to the molar concentration of antibody.

Optionally zinc is added in a molar concentration of at least 2 times, and preferably 3 times or 4 times the molar concentration of antibody.

Alternatively, a molar concentration of metallic cations is added with which to improve the functional activity of the antibody by at least 25%, preferably 50% or 60%, 70%, 80%, 100% and preferably 200 or 300%. Advantageously, the metallic cations exist in different forms. In one particular characteristic of the invention, the zinc ions may be in the form of zinc acetate, zinc bromide, zinc hydrochlorate, zinc chloride, zinc citrate, zinc gluconate, zinc hydroxycarbonate, zinc iodide, zinc L-lactate, zinc nitrate, zinc stearate, or zinc sulfate.

A further subject of the invention relates to class IgG3 antibodies, and more particularly the G3m(b) and G3m(g) allotypes, having a fixing site for a metallic cation, comprising His 310 and His 435 residues on its Fc region created by molecular engineering.

Under the invention, we have evidenced that metallic cations fix to class IgG1 antibodies on a site comprising the His 310 and His 435 residues, the His 433 and Asn 434 residues possibly also being involved in this fixing. However, class IgG3 antibodies of allotype G3m(b) or G3m(g) do not comprise said fixing site in the natural state. They have an arginine residue (Arg) at position 435. Therefore, under the invention, we create class IgG3 antibodies, by directed mutagenesis, having improved fixing of metallic cations with respect to non-modified antibodies, through the creation of a fixing site involving a His 435 residue substituting for the Arg 435 residue.

Therefore, these IgG3 antibodies comprise a fixing site for a metallic cation, in particular zinc, iron, copper, cadmium, cobalt, nickel, manganese, gallium, selenium, gold, platinum or palladium, which comprises the His 310 and His 435 residues, and advantageously also comprising the Asn 434

and/or His 433 residues. Advantageously, at least one of these histidine residues is replaced by at least one of the residues chosen from among cystein, aspartic acid and glutamic acid. These latter residues also have the capability of fixing said metallic cations. Preferably, the metallic cation is zinc, iron, copper or cadmium, and further preferably it is zinc.

According to one particular characteristic of the invention, the antibody has a metallic cation and more particularly a zinc atom, bound to one or more residues of the Fc region. In particularly advantageous manner, this IgG3 antibody has the capacity of fixing to FcγRIII and an improved functional activity with respect to the native antibody.

One subject of the invention is therefore the use of the IgG3 antibody, having a fixing site for a previously described metallic cation, to prepare a medicinal product intended to treat a pathology such as haemolytic disease of the new-born, a viral, bacterial or parasitic pathology, a pathology related to pathogenic agents or derived toxins listed as being particularly dangerous regarding bio-terrorism (classification of the Centers for Disease Control, CDC) in particular anthrax (*Bacillus anthracis*), botulism (*Clostridium botulium*), the plague (*Yersinia pestis)*, smallpox (*Variola major*), tularaemia (*Francisella tularensis*), viral haemorrhagic fevers (related to filoviruses: Ebola, Marburg and to arenaviruses - Lassa, Machupo), the *epsilon* toxin of *Clostridium perfringens*, brucellosis (*Brucella species)*, melioidosis (*Burkholderia mallei*), the toxin of castorbean (*Ricinus communis*).

A further subject of the invention is a pharmaceutical composition of therapeutic antibodies comprising divalent or trivalent cations and at least one excipient. Preferably, these metallic cations are zinc, iron, copper or cadmium or a mixture of several of these. In particularly advantageous manner, zinc is chosen which may be in the form of zinc acetate, zinc bromide, zinc hydrochlorate, zinc chloride, zinc citrate, zinc gluconate, zinc hydroxycarbonate, zinc iodide, zinc L-lactate, zinc nitrate, zinc stearate or zinc sulphate.

According to one particular characteristic, the antibodies contained in the composition have a metallic cation according to the invention bound to the His 310 and His 435 residues, the His 433 and Asn 434 residues possible also taking part in the fixing.

According to another particular characteristic of the invention, the antibodies of the pharmaceutical composition are class IgG3 antibodies created by molecular engineering as described previously. According to another preferred aspect of the invention, they are human IgGs or have a human Fc region.

Therefore the presence of said metallic cations in the composition improves the fixing of the therapeutic antibodies it contains to its receptors, in particular the FcγRIII receptors, the composition thereby having improved therapeutic activity.

A further subject of the invention is a pharmaceutical composition in which at least 50%, 60%, 70%, 80%, 90% or even 99 % of the antibodies have a divalent or trivalent metallic cation, a zinc ion in particular, bound to a site located in the Fc region. This may preferably be the fixing site comprising the amino acids His 310 and His 435, the amino acid Asn 434 and/or His 433 possibly also taking part in the fixing. According to another characteristic of the invention, this may be the fixing site comprising the amino acids His 268 and His 285. According to another characteristic, the 2 sites may be occupied by a metallic cation such as described in the invention.

The metallic cation is preferably one of those previously cited, in particular zinc, iron, copper or cadmium or a mixture of several of these, optionally in the forms already mentioned.

A further subject of the invention is a solution comprising a monoclonal antibody or monoclonal antibodies and a suitable quantity of divalent or trivalent metallic cations, in particular zinc ions in a molar concentration at least equal to the molar concentration of antibodies, this solution

being adapted for injection via intravenous, subcutaneous or intramuscular route.

The metallic cations may be any divalent or trivalent metallic cation, in particular zinc, iron, copper, cadmium, cobalt, nickel, manganese, gallium, selenium, gold, platinum or palladium or an analog. Preferably the cation is a zinc ion or zinc acetate, zinc bromide, zinc hydrochlorate, zinc chloride, zinc citrate, zinc gluconate, zinc hydroxycarbonate, zinc iodide, zinc L-lactate, zinc nitrate, zinc stearate or zinc sulphate.

A further subject of the invention is the use of zinc ions to improve the crystallisation of therapeutic antibodies, and more particularly of monoclonal IgGs, the zinc ions stabilising the Fc region of the antibodies. The addition of divalent cations and more particularly of zinc significantly increases the solubility of the Fc regions of IgGs by promoting crystalline contacts which facilitate the obtaining of the crystals required for structural studies.

One object of the invention is to provide a test with which to assess the efficacy of an antibody, comprising the study of 3D conformation, in particular of the domain involving the His 310, His 435, His 433 and/or Asn 434 residues of the Fc region such as shown in figure 1 or 2, or an assay of the zinc content of said antibodies, the presence of zinc being an indication of the efficacy of the antibody.

A further subject of the invention relates to an antibody having a modification of at least one its His 310 and His 435 residues.

According to one particular characteristic, the modification of the antibody is a mutation, in particular a substitution by an amino acid having low affinity for divalent or trivalent metallic cations. For example, the His 310 residue and/or His 435 residue may be substituted by a residue of lysine, alanine, glycine, valine, leucine, isoleucine, proline, methionine, tryptophan, phenylalanine, serine or threonine.

In particularly advantageous manner, the His 310 and His 435 residues are both substituted by lysine residues.

These mutants may be produced from any antibody which in the "natural" state, i.e. non-mutated, has a fixing site for metallic cations comprising the His 310 and His 435 residues. It may in particular be a IgG1, IgG3, allotype G3m(s) or G3m(st), IgG2 or IgG4.

These mutants have a significantly reduced capacity for activating FcγRIII.

In a second embodiment, the modification may be made using DEPC (diethyl pyrocarbonate), an agent which modifies histidines.

Advantageously, these antibodies are IgG1s, or at all events antibodies which in the "natural" state, i.e. non-mutated, have a fixing site for metallic cations comprising the His 310 and His 435 residues.

These antibodies have a reduced functional activity with respect to the same non-modified antibody. However, they maintain their ability to fix the antigen and FcγRIII.

Therefore, the invention provides antibodies having low ADCC activity, which are of particular therapeutic interest to replace IgG4s, or to prevent graft rejections. Double mutant antibodies of the invention may also be used as anti-tetanus, anti-diphtheria antibodies or those directed against pathogenic agents or derived toxins listed as being particularly dangerous in the event of bio-terrorism (classification of the Centers for Disease Control, CDC), in particular anthrax (*Bacillus anthracis*), botulism (*Clostridium botulium*), the plague (*Yersinia pestis*), smallpox (*Variola major*), tularaemia (*Francisella tularensis*), viral haemorrhagic fevers (related to filoviruses: Ebola, Marburg and to arenaviruses - Lassa, Machupo), the *epsilon* toxin of *Clostridium perfringens*, brucellosis (*Brucella species*), melioidosis (*Burkholderia mallei*) the toxin of castorbean (*Ricinus communis*).

Therefore a further subject of the invention concerns the use of antibodies modified as described above, and therefore

having low functional activity, for the preparation of a medicinal product intended to prevent graft rejection or for the treatment of a pathology chosen from among tetanus, diphtheria, or caused by a pathogenic agent or derived toxin listed as being particularly dangerous in the event of bio-terrorism (classification of the Centers for Disease Control, CDC), in particular anthrax (*Bacillus anthracis*), botulism (*Clostridium botulium*), the plague (*Yersinia pestis*), smallpox (*Variola major*), tularaemia (*Francisella tularensis*), viral haemorrhagic fevers (related to filoviruses: Ebola, Marburg and to arenaviruses - Lassa, Machupo), the *epsilon* toxin of *Clostridium perfringens*, brucellosis (*Brucella species*), melioidosis (*Burkholderia mallei*) the toxin of castorbean (*Ricinus communis*).

Finally, antibodies having impaired functional activity as previously described are also used for the preparation of a medicinal product to replace IgG4s.

By way of example, the antibodies described in the invention, in particular the antibodies having improved functional activity due to metallic cations, or modified antibodies whose functional activity is impaired, or the compositions or solutions of the invention may be chosen from among anti-Ep-Cam, anti-KIR3DL2, anti-EGFR, anti-VEGFR, anti-HER1, anti-HER2, anti-GD, anti-GD2, anti-GD3, anti-CD20, anti-CD23, anti-CD25, anti-CD30, anti-CD33, anti-CD38, anti-CD44, anti-CD52, anti-CA125 and anti-ProteinC, anti-HLA-DR, the antivrials: HBV, HCV, HIV and RSV and more particularly among the antibodies in table I below:

Table I

Antibody name & trade-mark	Company	Target	Indication
Edrecolomab PANOREX	Centocor	anti-Ep-CAM	colorectal cancer
Rituximab RITUXAN	Idec Licensed to Genentech/Hoffman La Roche	anti-CD20	B cell lymphoma thrombocytopaenia purpura

Trastuzumab HERCEPTIN	Genentech Licensed to hoffman LA Roche/Immunogen	anti-HER2	ovarian cancer
Palivizumab SYNAGIS	Medimmune Licensed to Abbott		RSV
Alemtuzumab CAMPATH	BTG Licensed to Schering	anti-CD52	Leukaemia
Ibritumomab tiuxetan ZEVALIN	IDEC Licensed to Schering	anti-CD20	NHL
Cetuximab IMC-C225	Merck/BMS/ Imclone	anti-EGFR	cancers
Bevacizumab AVASTIN	Genentech / Hoffman La Roche	anti-VEGFR	cancers
Epratuzumab	Immumedics/ Amgen	anti-CD22	cancers: non-Hodgkins lymphoma
Hu M195Mab	Protein Design Labs	anti-CD33	cancers
MDX-210	Immuno-Designed Molecules	ND	cancers
BEC2 Mitumomab	Imclone	anti-GD3	cancers
Oregovomab OVAREX	Altarex	anti-CA125	ovarian cancer
Ecromeximab KW-2971	Kyowa-Hakko	anti-GD3	malignant melanoma
ABX-EGF	Abgenix	EGF	cancers
MDX010	Medarex	anti-CD4R	cancers
XTL 002	XTL Biopharmaceuticals	ND	antiviral: HCV
H11SCFV	Viventia Biotech	ND	cancers
4B5	Viventia Biotech	anti-GD2	cancers
XTL 001	XTL Biopharmaceuticals	ND	antiviral: HBV
MDX-070	MEDAREX	anti-PSMA	prostate cancer
TNX-901	TANOX	anti-IgE	allergies
IDEC-114	IDEC	ProteinC inhibition	non-Hodgkins lymphoma

For example, the invention relates to a solution in the form of a concentrate with an antibody concentration ranging from 0.1 to 50 mg/mL, or 1 to 25 mg/mL which may be formulated for administering via IV route. The solute may for example comprise: 9.0 mg/mL sodium chloride, 7.35 mg/mL dehydrated sodium citrate and 0.7 mg/mL polysorbate-80 in sterile water. In this solute or concentrate 0.1 to 50 mg/mL or 1 to 20 mg/mL of a cation are also added, the concentration of said cation being equal to 1, 2, 3, 4, or 5 times the concentration of the antibody i.e. 0.1 to 250 mg/mL or 1 to 100 mg/mL. This concentrate may be injected into a serum bag or liquid perfusion bag in order to obtain the dose it is desired to administer, while maintaining the same cation content with respect to the antibody content.

The invention also relates to a sterile, freeze-dried powder in a container, which can be reconstituted with sterile water just before injecting and comprising the suitable quantity of antibody, the quantity of said cation as per the invention being 1,2,3,4 or 5 times greater than the quantity of antibody.

This powder can be reconstituted for IV or subcutaneous injection. In the latter case the powder may comprise 10 to 500 mg antibody and a quantity of said cation according to the invention 1,2,3,4 or 5 time greater than the quantity of antibody, i.e. 10 to 500 mg for example. Excipients may be added such as sucrose, an amino acid, polysorbate.

It is to be understood that the invention preferably concerns the above-described compositions in which the cation content is at least equal to the antibody content, but also concerns any composition in which a quantity of cation is added (zinc, iron, copper, cadmium or analog or their mixture) that is less than said equimolar quantity e.g. from 0.1 to 0.99 molar (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 or 0.9 molar) but at all times sufficient to improve by at least 10% or 25%, or 50% or even 100% the efficacy and/or functional properties of the antibody.

Other characteristics and advantages of the invention will be described in the following examples which are to be considered as illustrations not limiting the scope of the invention.

In order to acquire new data on the structure-function relationships of the Fc region of antibodies, the applicant crystallized Fc fragments of the monoclonal antibody EMAB5, expressed in the YB2/0 cell line, in the presence or absence of zinc. X-ray diffraction analysis (XR) of the crystallized Fc fragments was performed; part of this study is shown in examples 1 and 2 below. Example 3 shows the effect of a chemical modification of the histidines on Fc activity, and example 4 the effect of a double mutation of the histidine 310 and 435 residues into lysines.

DESCRIPTION OF THE FIGURES

- Figure 1: Schematic showing the position of the Zn2+ ions in the vicinity of the Fc fragment of the monoclonal antibody EMAB(2Å)

- Figure 2: Detail of electronic density map in the vicinity of the histidine 310 and 435 residues

- Figure 3: Superimposition of structures of the Fc fragment of the antibody EMAB5 obtained in the absence (grey) and presence (white) of Zn2+ ions.

- Figure 4: Fixing of the DEPC-modified EMAB5 antibody to the FcγRIII receptor. This figure, along the abscissa axis, shows the fixing of antibodies to erythrocytes, and along the ordinate axis the fixing of CD16 (FcγRIII) present on the surface of CD16 Jurkat cells.(♦) control antibody AD1; (■) control EMAB5; (▲) EMAB5 NRF; (●) EMAB5 RF

- Figure 5: Activation by the DEPC-modified EMAB5 antibody of the FcγRIII receptor present on the Jurkat CD16 cells.

This figure shows the quantity, expressed in pg/ml, of IL-2 secreted by the Jurkat CD16 cells whose CD16 receptor was activated by the control EMAB5 antibody (♦) and the fractions

separated on protein A after modification of the EMAB5 antibody by DEPC: NRF (●), RF (■).

- Figure 6: Effect of modification by DEPC on the ADCC activity of the monoclonal antibody EMAB5.

This figure shows the lysis percentage of erythrocytes Rh(D+) induced by the control EMAB5 monoclonal antibody and by the 2 RF and NRF fractions, separated on Sepharose-protein A gel, of the DEPC- modified antibody.

- Figure 7: Measurement of Fc fixing of double-mutant antibodies His310-435Lys to the FcγRIII receptor. The antibodies 1C7, 2H11, 4G5 and 4H10 are the mutated antibodies. Antibodies 16D11, 11G5 and 6H11 are not mutated. This figure, along the abscissa axis, shows the fixing of the antibodies to the erythrocytes, and along the ordinate axis the fixing of CD16 (FcγRIII) present on the surface of Jurkat CD16 cells. The figure groups together the results obtained with the supernatants containing the double mutated antibodies (solid plotted lines) and those containing non-mutated antibodies (dotted plotted lines).

- Figure 8: Study of IL-2 secretion induced by anti-Rh(D) monoclonal antibodies whether mutated or not. This figure shows the percentage of IL-2 secreted by native anti-Rh(D) monoclonal antibodies (n=3 clones) and those carrying the double mutation His310-435Lys (n=4 clones). The results are expressed as a percentage with respect to a purified, control EMAB5 antibody.

- Figure 9: Influence of imidazole on the fixing of the EMAB5 monoclonal antibody to the CD16 receptor (FcγRIII).

- Figure 10: Study by flow cytometry of the fixing of antibodies carrying the double mutation His310-435Lys to the CD16 receptor.

EXAMPLES

In order to acquire new data on the structure-function relationships of the Fc region of antibodies, the applicant crystallized Fc fragments of the EMAB5 monoclonal antibody, expressed in the YB2/0 cell line, in the presence or absence

of zinc. X-ray diffraction analysis of the crystallized Fc fragments was performed; part of this study is shown in examples 1 and 2 below. Example 3 shows the effect of a chemical modification of the hisidines on Fc activity and example 4 shows the effect of a double mutation of histidine residues 310 and 435 into lysins.

Monoclonal antibody:

EMAB5. This is a human IgG1(κ)directed against the antigen Rh(D), produced in the YB2/0 cell line (rat myeloma, ATCC line n°CRL 1662) adapted for culture in a serum-free medium.

- Purification: EMAB5 was purified by affinity chromatography on Sepharose-protein A.
- Glycannic analysis: by HPCE-LIF, it was shown that the major part of the structure is an oligosaccharide of biantenna type containing approximately 25% fucose.
- Biological activity: the ADCC activity of EMAB5 is at least equal to that of the control anti-Rh(D) polyconal antibody, WinRho(Cangène).

Preparation of the Fc fragment:

- Hydrolysis conditions: the purified EMAB5 antibody is dialysed overnight against 50 mM Tris buffer, pH 8.0. The antibody solution, adjusted to 50 mM CaCl2 and 10 mM cystein is incubated 30 min. at 37°C before adding the trypsin solution (1 mg/ml) in an enzyme-to-substrate ratio of 1/25. After 5 h incubation at 37°C the reaction is stopped by the addition of diisopropyl fluorophosphate (final 1 mM). The hydrolysate is dialysed overnight against 50 mM imidazole buffer, pH 7.8
- Purification of the Fc fragment: the dialysed hydrolysate is contacted with Affarose-protein L to the proportion of 1 ml gel per 3.6 mg antibody. After 4h incubation at room temperature under stirring, the gel is mounted in a column and washed with the 50 mM imidazole buffer, pH 7.8. The effluent and the washing buffer containing the Fc fragments

are grouped together, concentrated by centrifuging on Vivaspin 20 following the manufacturer's instructions.

EXAMPLE 1: Presence of zinc ions bound to Fc fragments

Crystallogenesis:

After testing, the following crystallisation conditions are chosen: the solution of Fc fragments, in 2 mg/ml 50 mM Imidazole buffer pH 7.8, is brought to 10% monomethyl polyethylene glycol 5000, 100 mM sodium cacodylate, 0.1 mM zinc chloride pH 5.1 by sitting drop vapour diffusion at 17°C.

Collection of diffraction data and structure determination:

The crystal obtained was subjected to XR analysis at the ESRF in Grenoble and the collected data were processed using DENZO and SCALEPACK programmes (Otwinowski and Minor, 1997).
The structure is resolved and fine-tuned to 2.3 Å (Fig. 1) using the series of CCP4 programmes.

Results

This crystal of the Fc fragment of EMAB5 belongs to the $C222_1$ space group with one fragment per asymmetric unit. Lattice parameters are the following: a=50.2 Å; b=147.7 Å; c=75.6 Å; $\alpha = \beta = \gamma = 90°$.
The three dimensional structure (schematised in figure 1) allows evidencing of the presence of zinc ions (in white) close to the CH2 and CH3 domains (in grey). The electronic density map in figure 2 shows the zinc ion bound to the histidine residues 310 (CH2) and 435 (CH3). Another zinc ion is bound to histidine 268 of a Fc and to histidine 285 of a symmetrical Fc. The third is located close to histidine 433.

EXAMPLE 2: Effect of metallic cations on the conformation of Fc fragments

In this example, the crystals obtained all belong to space group P2(1)2(1)2(1)with two chains per asymmetric unit, and Fc opening is characterized by the distances between the proline

329 residues of chains A and B and between the mannose 4 residues of chains A and B.

Crystallized in the absence of a metallic ion, the Fc fragments of the EMAB5 antibody are characterized by a distance between prolines 329 of 32.53 Å, and an inter-mannose 4 distance of 17.61 Å. When a metal ion is added to the crystallisation solution (see Table II), these characteristic distances are increased.

Table II: Main characteristics of the crystals of the Fc fragment of the monoclonal antibody EMAB5 in the space group P2(1)2(1)2(1)

Code	Metal in cryst.sol.	Resolution (Å)	Lattice parameters (Å;angles=90 Å)	D Pro 329 (Å)	D Man 4 (Å)
Fc1_nat	No	3.29	48.982 75.498 145.032	32.53	17.61
Fc1_Zn	0.3mM ZnCl2	3.2	49.716 75.516 150.298	37.12	20.34
Fc1_Gd	2mM GdCl3	2.7	49.816 76.068 151.156	37.4	20.75
Fc1_Cu	0.3mM Cu++Ac	3.15	49.462 74.985 148.928	35.87	19.79
Fc1_Fe	0.1mM Fe^{3+}	3.475	49.056 74.937 148.148	35.78	19.61
Fc1_Cd	0.2mM CdCl2	2.894	49.253 75.079 147.953	36.18	19.2

Figure 3 shows the superimposition of the main chains of the structures obtained in the absence (in grey) and in the presence (white) of zinc in the crystallisation solution. The addition of a metal salt to the Fc solution therefore promotes a so-called open conformation, a conformation close to that of the Fc bound to the FcγRIII receptor.

EXAMPLE 3: Modification of histidine residues by DEPC

Diethylpyrocarbonate (DEPC) is a reagent which has been widely used to modify and examine the role of histidine

residues present in proteins (Miles, 1977). Firan *et al* (2001) showed that human IgG1s treated with DEPC lose their capacity to fix the FcRn receptor which is involved in the transfer of maternal IgGs to the foetus. DEPC acts by substitution of a nitrogen group present on the imidazole cycle of the histidine, thereby transforming the histidine residue into 3-carboethoxy histidine.

1. Modification by DEPC

The monoclonal antibody EMAB5, dialysed against 0.1M sodium acetate buffer pH 6.0, is contacted with DEPC to the proportion of 70 µg DEPC/mg IgG. After 30 minutes' incubation at room temperature, the reaction is stopped with the addition of imidazole (final 0.2 mg/ml).

After desalting in 20 mM sodium phosphate buffer, 50 mM NaCl, pH 7.2 the modified monoclonal antibody is fractionated by affinity chromatography on Sepharose-protein A. One fraction of the modified monoclonal antibody is not retained on the affinity gel and forms the non-retained fraction or NRF. The retained fraction on the gel called RF is eluted with 0.1M Glycine-HCl buffer, pH 2.8. The two fractions so obtained are dialysed against 20 mM sodium phosphate buffer, 50 mM NaCl, pH 7.2 concentrated on Vivaspin following the manufacturer's instructions and stored at 4°C for no more than 15 days.

The control antibody, used as reference, underwent the same treatment with the exception of DEPC which was replaced by an identical volume of ethanol.

2. Measurement of antibody Fc fixing to the FcγRIII receptor using the CFC test.

In order to verify the integrity of the antibodies treated with DEPC, the antibodies are subjected to a test called the CFC test which estimates the capacity of the antibodies firstly to fix the antigen against which they are directed and secondly to fix the FcγRIII receptor (CD16) expressed on the surface of the CD16 Jurkat cell line.

The wells of a microtitration plate are coated with papain-treated erythrocytes Rh(D+). The anti-Rh(D) antibodies, diluted to concentrations varying from 7.8 to 500 ng/ml in IMDM + 2.5% foetal calf serum (FCS), are deposited in parallel on two microtitration plates previously coated with the erythrocytes. After 90 min. incubation at 37°C the wells are washed.

One of the plates, used to detect the IgGs fixed to the erythrocytes, is incubated in the presence of mouse anti-human Fcγ antibody labelled with alkaline phosphatase (Jackson ImmunoResearch Laboratories).

In the other plate the CD16 Jurkat cells are added diluted to a concentration of 2×10^6 cells/ml in IMDM + 1 % FCS. After 15 min. contact at 37°C the plate is centrifuged gradually increasing speed and centrifuging time until the negative controls are negated. By negative controls are meant the antibodies which fix to the erythrocytes immobilised in the wells of the microtitration plate but which do not fix the FcγRIII receptor present on the surface of the CD16 Jurkat cells. In the wells containing the negative control, the CD16 Jurkat cells, after centrifuging, form a cluster in the centre of the well whereas in the wells containing a positive control the CD16 Jurkat cells coat the well.

After centrifuging, well reading is carried out and a score is given in relation to the spreading of CD16 Jurkat cells in the well.

3. Measurement of activation of the FcγRIII receptor

The activation test of CD16 Jurkat cells measures the secretion of interleukin-2 (IL-2) induced by the fixing of the Fc of the antibodies onto the FcγRIII receptor (CD16) after binding of the Fab to its antigen, present on the target cell. The rate of IL-2 secreted by the CD16 Jurkat cells is proportional to the activation of the CD16 receptor.

In a 96-well microtitration plate, 50 µl of antibody dilutions, 50 µl of a suspension of erythrocytes at 6.10^5/ml, 50 µl of a suspension of CD16 Jurkat cells at 1.10^6/ml and 50

µl of PMA solution at 40 ng/ml are successively added. All the dilutions are made in IMDM culture medium containing 50% FCS.

After 16 hours' incubation at 37°C and 7% CO2 the microtitration plate is centrifuged and the quantity of IL-2 contained in the supernatant is assayed using a commercial kit (Duoset, R&D). The rates of secreted Il-2 are expressed in pg/ml.

The results are expressed as a % of CD16 activation, the rate of IL-2 secreted in the presence of the control monoclonal antibody being considered equal to 100%.

4. Measurement of ADCC activity

The ADCC technique (Antibody-Dependent Cell-mediated Cytotoxicity) is used to evaluate the capacity of the antibodies to induce the lysis of erythrocytes Rh(D+) in the presence of effector cells (mononucleated cells or lymphocytes).

Briefly, the erythrocytes of a globular RhD(+) concentrate are treated with papain (1mg/ml, 10 min at 37°C) then washed in 0.9% NaCl. The effector cells are isolated from a pool of at least 3 buffy-coat, by centrifuging on Ficoll (Amersham Biosciences), followed by an adherence step in the presence of 25% FCS, in order to obtain a lymphocyte/monocyte ratio in the order of 9. In a microtitration plate (96-well) the following are deposited per well: 100 µl of a dilution of purified anti-Rh(D) antibody(from 9.3 to 150 ng/ml), 25 µl Rh(D+) papain-treated erythrocytes 4 x 10^7, 25 µl of effector cells 8 x $.10^7$ and 50 µl polyvalent IgG (Tégéline, LFB) at the usual concentrations of 2 and 10 mg/ml. The dilutions are made in IMDM containing 0.25% FCS. After incubation overnight at 37°C the plates are centrifuged then the haemoglobin released in the supernatant is measured via its peroxydase activity in the presence of a chromogenic substrate 2,7-diaminofluorene (DAF). The results are expressed as a lysis percentage, 100% corresponding to total lysis of the erythrocytes in NH_4Cl (100% control) and 0% to the reaction mixture without antibody

(0% control). Specific lysis is calculated as a percentage using the following formula:

$$\frac{\text{(sample OD - 0\% control OD) X 100}}{\text{100\% control OD - 0\% control OD}} = \text{\% ADCC}$$

Results:

After treatment with DEPC in accordance with the above-described conditions, approximately 20% of the molecules of the monoclonal antibody EMAB5, forming the NRF, lose their capacity to fix to the Sepharose-protein A gel. Bearing in mind that histidine 435 is an essential amino acid for the fixing of IgGs to protein A, it appears probable that the IgGs of the NRF differ from the RF fraction, retained on the Sepharose-protein A gel, through the modification of the His435 residue.

In the test to measure fixing to the CD16 receptor in the presence of antigen, the NRF fraction shows the same capacities as the RF fraction, capacities which are identical to those of the control antibody (Fig.4). In addition, the fixing of the different fractions of the monoclonal antibody EMAB5, whether DEPC modified or not, is much higher than that of the monoclonal antibody AD1 used in this test as negative control. Therefore the modification of histidine residues by DEPC does not induce any change in the capacity of the monoclonal antibody EMAB5 to fix to the Rh(D+) erythrocytes or to the CD16 receptor present on the surface of the CD16 Jurkat cell line.

On the other hand, the functional activity of the monoclonal antibody EMAB5, modified by DEPC, is largely reduced. The secretion of IL-2 of the CD16 Jurkat cell line induced by the RF and NRF fractions of the monoclonal antibody EMAB5 modified by DEPC respectively represents 42.8% and 19.5% of the secretion induced by the control antibody (Fig.5). The results of ADCC activity, expressed as a % of actual lysis, show that the activity of the NRF fraction is lower than that of the RF fraction (Fig.6). Also, the RF fraction shows

reduced ADCC activity with respect to the control antibody when the quantity of antibody added to the well is smaller (<20 ng/ml) and the quantity of polyvalent IgGs is 2.5 mg/ml.

To conclude, even though they maintained their capacity to fix the antigen and FcγRIII receptor (CD16), the fractions of DEPC-modified EMAB5 monoclonal antibody show reduced functional activity (ADCC, induction of cytokin secretion). This reduced activity is more marked for the NRF fraction, a fraction which most probably has a modification of the His435 residue.

These results therefore show that conservation of the His435 residue is important for preserving the functional activity of IgG1-type antibodies.

EXAMPLE 4: Functional activity of the antibodies carrying the double mutation His310-435Lys.

The modification of histidine residues by a chemical agent (DEPC or other) does not make it possible to control either the degree or the locations of these modifications. Therefore, the two histidine residues His310 and His435 which play an essential role in the binding of the zinc cation to the CH2-CH3 interface of IgG1s, are substituted by lysine residues, via directed mutagenesis.

1. Obtaining an anti-Rh(D) antibody carrying the double mutation His310-435Lys.

The expression vector containing the cDNA encoding the sequence of amino acids of the heavy chain of the anti-Rh(D) EMAB5 antibody, was used as matrix to conduct directed double mutagenesis by PCR ("PCR-based site-directed mutagenesis"). The four following nucleotide substitutions were made:

- C1229A and C1301G to change the His338 residue to Lys (position 310 as per Kabat numbering) i.e. CAC → AAG;
- C1674A and C1676G for mutation of the His463 residue to Lys (position 435 as per Kabat numbering), i.e. CAC → AAG

The mutated sequence was sequenced and the sequencing results are given in Table III.

Table III: Oligonucleotide and polypeptide sequences of the double mutated heavy chain of the monoclonal antibody EMAB5.

The His338 and His461 residues of the heavy chain of the monoclonal antibody EMAB5, which correspond to residues His310 and His435 as per Kabat numbering, were substituted by lysine residues.

Sequence of the cDNA of the double mutant His310-435Lys (SEQ ID No1)

atggagtttgggctgagctgggttttcctcgttgctcttttaagaggtgtccagtgtcaggtgcagctggtggagtctgggggag
gcgtggtccagcctgggaggtccctgagactctcctgtacagcctctggattcaccttcaaaaactatgctatgcattgggtcc
gccaggctccagccaaggggctggagtgggtggcaactatatcatatgatggaaggaatatacaatatgcagactccgtgaa
gggccgatgcaccttctccagagacaattctcaggacaccctgtatctgcaactgaacagcctcagaccggaggacacggct
gtgtattactgtgcgagacccgtaagaagccgatggctgcaattaggtcttgaagatgcttttcatatctggggccaggggaca
atggtcaccgtctcttcagcctccaccaagggcccatcggtcttcccctggcaccctcctccaagagcacctctgggggcac
agcggccctgggctgcctggtcaaggactacttccccgaaccggtgacggtgtcgtggaactcaggcgccctgaccagcgg
cgtgcacaccttcccggctgtcctacagtcctcaggactctactccctcagcagcgtggtgaccgtgccctccagcagcttgg
gcacccagacctacatctgcaacgtgaatcacaagcccagcaacaccaaggtggacaagaaagttgagcccaaatcttgtg
acaaaactcacacatgcccaccgtgcccagcacctgaactcctgggggggaccgtcagtcttcctcttcccccaaaacccaa
ggacaccctcatgatctcccggacccctgaggtcacatgcgtggtggtggacgtgagccacgaagaccctgaggtcaagttc
aactggtacgtggacggcgtggaggtgcataatgccaagacaaagccgcgggaggagcagtacaacagcacgtaccgtgt
ggtcagcgtcctcaccgtcctgaagcaggactggctgaatggcaaggagtacaagtgcaaggtctccaacaaagccctccc
agcccccatcgagaaaaccatctccaaagccaaagggcagccccgagaaccacaggtgtacaccctgcccccatcccggg
atgagctgaccaagaaccaggtcagcctgacctgcctggtcaaaggcttctatcccagcgacatcgccgtggagtgggaga
gcaatgggcagccggagaacaactacaagaccacgcctcccgtgctggactccgacggctccttcttcctctacagcaagct
caccgtggacaagagcaggtggcagcaggggaacgtcttctcatgctccgtgatgcatgaggctctgcacaacaagtacac
gcagaagagcctctccctgtctccgggtaaatag

Peptide sequence of the double mutant His310-H435Lys (SEQ ID No2):

```
1   MEFGLSWVFL VALLRGVQCQ VQLVESGGGV VQPGRSLRLS CTASGFTFKN
51  YAMHWVRQAP AKGLEWVATI SYDGRNIQYA DSVKGRCTFS RDNSQDTLYL
101 QLNSLRPEDT AVYYCARPVR SRWLQLGLED AFHIWGQGTM VTVSSASTKG
151 PSVFPLAPSS KSTSGGTAAL GCLVKDYFPE PVTVSWNSGA LTSGVHTFPA
201 VLQSSGLYSL SSVVTVPSSS LGTQTYICNV NHKPSNTKVD KKVEPKSCDK
251 THTCPPCPAP ELLGGPSVFL FPPKPKDTLM ISRTPEVTCV VVDVSHEDPE
301 VKFNWYVDGV EVHNAKTKPR EEQYNSTYRV VSVLTVLKQD WLNGKEYKCK
351 VSNKALPAPI EKTISKAKGQ PREPQVYTLP PSRDELTKNQ VSLTCLVKGF
401 YPSDIAVEWE SNGQPENNYK TTPPVLDSDG SFFLYSKLTV DKSRWQQGNV
451 FSCSVMHEAL HNKYTQKSLS LSPGK*
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The YB2/0 cells, co-transfected by electroporation with the mutated vector EMAB5-H-K338-K463-1 and the vector EMAB5-dhfr-K-SpeI coding for the light chain of the EMAB5 antibody, are cultured in RPMI medium to which is added 5% dialysed FCS, 0.5% G418 and 25 mM Methotrexate (MTX). The clones secreting the highest rates of human IgGs are cultured in 24-well plates in MTX-free medium. The supernatants, harvested after 7 days' culture, are used to perform the tests described below.

2. Measurement of antibody Fc fixing to the FcγRIII receptor(CFC).

This test is performed on the culture supernatants, the human IgG content in the supernatants being determined by ELISA assay.

The wells of a microtitration plate are coated with papain-treated Rh(D+) erythrocytes. The culture supernatants containing the native or mutated anti-Rh(D) antibodies and diluted to concentrations varying from 7.8 to 500 ng/ml in IMDM + 2.5% FCS, are deposited in parallel on two microtitration plates previously coated with erythrocytes. After 90 min. incubation at 37°C the wells are washed.

One of the plates, used to detect the IgGs fixed to the erythrocytes, is incubated in the presence of a mouse anti-

human Fcγ antibody labelled with alkaline phosphatase (Jackson ImmunoResearch Laboratories).

In the other plate, the CD16 Jurkat cells are added after 15 min. contact at 37°C, the plate is centrifuged gradually increasing speed and centrifuging duration until the negative controls are negated. After centrifuging, reading of the wells is conducted and a score is given in relation to the spreading of the CD16 Jurkat cells in the well.

3. Measurement of CD16 receptor activation

In a 96-well microtitration plate, 50 µl of dilutions of culture supernatants containing native or mutated anti-Rh(D) antibodies, 50 µl of a suspension of erythrocytes at 6.10^5/ml, 50 µl of a suspension of CD16 Jurkat cells at 1.10^6/ml and 50 µl of a PMA solution at 40 ng/ml are successively added. All the dilutions are made in IMDM culture medium containing 5% FCS.

After 16 hours' incubation at 37°C and 7% CO2, the microtitration plate is centrifuged and the quantity of IL-2 contained in the supernatant is assayed with a commercial kit (Duoset, R&D). The rates of secreted IL-2 are expressed in pg/ml.

The results are expressed as % CD16 activation, the percentage of IL-2 secreted in the presence of the control monoclonal antibody being considered as 100%.

The different tests were conducted on culture supernatants containing the anti-Rh(D) monoclonal antibodies, whether mutated or not. The results of the CFC test show that the double mutation His310-435Lys does not induce any change in the fixing of the antibody to the FcγRIII receptor, carried by the CD16 Jurkat cell line (Fig.7). Whereas the non-mutated clone 6H11 (negative control) shows reduced fixing to the FcγRIII receptor, the mutated clones 1C7, 2H11, 4G5 and 4H10 fix the FcγRIII recpetor in similar manner to the non-mutated clones 16D11 and 11G5 (positive controls).

CD16 activation was performed on the culture supernatants of 4 mutated clones cited above and of 3 non-mutated clones

(16D11, 11G5 and 24G9). The results show the mean rates (± standard deviation) of IL-2 secreted by the CD16 Jurkat cells in the presence of non-mutated clones (Native) and of mutated clones (His310-435Lys). The mutated antibodies induce largely reduced secretion of IL-2 with respect to the quantity induced by the native antibodies (Fig.8). Therefore the mutated antibodies show a 50% drop in the capacity to activate the FcγRIII recpetor.

EXAMPLE 5: Flow cytometry study on the fixing of monoclonal antibodies, whether mutated or not, onto to the FcγRIII receptor

The influence of imidazole and the impact of His310-435Lys mutations on the fixing of antibodies to the FcγRIII receptor (CD16) present on the surface of the CD16 Jurkat cells, were assessed by flow cytometry.

1. Effect of imidazole

5.10^5 CD16 Jurkat cells are incubated 30 minutes in the presence of different concentrations of monoclonal antibody EMAB5 diluted in PBS buffer containing 0.5% bovine albumin serum (BAS) or 0.5% PBS-BAS buffer supplemented with 50 mM imidazole. Then the cells are washed in 0.5% PBS-BAS buffer and incubated in the presence of mouse anti-human IgG (H+L) F(ab')2 labelled with FITC (Jackson ImmunoResearch Laboratories). After 30 minutes' incubation, the cells are washed as previously and fixing of the EMAB5 antibody is analysed by flow cytometry using FACScalibur 4CA and the Cell Quest Pro programme (Becton Dickinson).

2. Fixing of the double mutated antibody

5.10^6 CD16 Jurkat cells are incubated for 30 minutes in 0.5% PBS-BAS buffer in the presence of different concentrations of monoclonal antibodies contained in the culture supernatants. The wells are then washed in 0.5% PBS-BAS buffer and incubated in the presence of mouse anti-human IgG (H+L) F(ab')2 labelled with FITC. After 30 minutes'

incubation, the cells are washed as previously and the fixing of the antibodies is analysed by flow cytometry using FACScalibur 4CA and the Cell Quest Pro programme (Becton Dickinson).

The results of the fixing of the EMAB5 antibody to the CD16 receptor (FcγRIII) present on the surface of the CD16 Jurkat cells in the presence or absence or imidazole are given in Fig. 9

The addition of 50 mM imidazole to the incubation buffer of the antibody with the cells causes reduced fixing of the antibody translating as a significant drop in the percentage of labelled cells; at an antibody concentration of 1.5 µg/ml, the presence of imidazole induces a 40% reduction in the number of labelled cells.

Imidazole is a reagent which has the property of fixing cations. Therefore, by depriving the incubation medium of cations through the addition of imidazole, the fixing of the antibody onto the CD16 receptor is reduced.

The fixing onto the FcγRIII receptor of the CD16 Jurkat cells of 3 monoclonal antibodies contained in the culture supernatants is shown Fig. 10.

Therefore, the results expressed as a percentage of labelled cells in relation to the quantity of added antibody, show that the 4G5 and 4H10 antibodies which have the double mutation His310-435Lys, fix themselves to the CD16 Jurkat cells to a significantly lesser extent than the 24G9 clone which is the non-mutated control antibody.

These results clearly illustrate that the fixing of antibodies to the CD16 receptor is affected by the absence of cations. Conversely, the presence of cations should improve the fixing of antibodies to the receptor and thereby improve the cytotoxic activity of the antibody.

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